Immunology 1985 **55** 547

A comparative study on the internal defence system of juvenile and adult *Lymnaea stagnalis*

R. DIKKEBOOM, W. P. W. VAN DER KNAAP, ELISABETH A. MEULEMAN & T. SMINIA*
*Laboratory of Medical Parasitology and *Department of Histology, Faculty of Medicine, Free University, Amsterdam, The Netherlands*

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Summary. The immunological immaturity of juvenile specimens of some snail species, e.g. *Lymnaea stagnalis*, may contribute to their greater susceptibility to infection by schistosome parasites. In a comparison between juvenile and adult specimens of the pond snail *L. stagnalis*, we have shown that the blood cells (amoebocytes) of juvenile snails are less efficient at phagocytosing: fewer amoebocytes are competent and the average number of particles engulfed per cell is lower. This functional immaturity seems to correlate with morphological immaturity of the amoebocytes. Opsonic and haemagglutinating activities are low in juvenile snail plasma, but much higher in adult plasma. Finally, however, the initial rate at which injected bacteria are eliminated from the circulation seems only slightly slower in juvenile snails than it is in adults.

INTRODUCTION

Gastropod molluscs possess a very effective immune system which can dispose of a variety of foreign particles and organisms (for review see Sminia, 1981). Snails like *Biomphalaria glabrata*, *Helix pomatia* and *Lymnaea stagnalis* are able to clear large doses of foreign injected material rapidly from their haemolymph (Tripp, 1961; Bayne, 1980; Renwrantz *et al.*, 1981; Van der Knaap *et al.*, 1981). Some organisms, however, e.g. schistosome parasites, are not destroyed by the internal defence system of certain snails and can use their host as a place for nutrition, growth and multiplication. Thus, susceptible strains of e.g. *B. glabrata* are obligate intermediate hosts for *Schistosoma mansoni* which causes schistosomiasis in the definitive host, man.

One of the snail–parasite combinations in which interactions between schistosomes and their intermediate molluscan hosts can well be studied is *Lymnaea stagnalis–Trichobilharzia ocellata* (e.g. Meuleman, Huyer & Mooij, 1984b). In this combination, as in others, juvenile snails are more susceptible to the parasite than are adults (Meuleman, 1980; Meuleman, Huyer & Luub, 1982). This might be because juvenile snails have a less well developed, and thus less competent, immune system. The internal defence system of adult *L. stagnalis* is well developed, and consists of a cellular component (Sminia, 1972; Sminia, Van der Knaap & Kroese, 1979b) and a humoral one (Sminia, Van der Knaap & Edelenbosch, 1979a; Van der Knaap *et al.*, 1982). However, very little is known about the immune system of juvenile snails. More knowledge about the ontogeny of the internal defence system of *L. stagnalis* could help to explain the occurrence of age-related susceptibility.

Correspondence: Dr Ronald Dikkeboom, Laboratory of Medical Parasitology, Faculty of Medicine, Free University, PO Box 7161, 1007 MC Amsterdam, The Netherlands.

In a previous paper (Dikkeboom *et al.*, 1984), a part of the cellular defence system (the circulating amoebocytes) of juvenile *L. stagnalis* was compared with that of adults and was found to be underdeveloped from a morphological point of view. In the present study, some functional aspects of the immune system of both age groups are compared: *in vitro* phagocytosis by amoebocytes, humoral defence activities (namely opsonization and agglutination) and the elimination of bacteria *in vivo*.

MATERIALS AND METHODS

Snails

Laboratory-bred specimens of the pond snail *Lymnaea stagnalis* were obtained from the Biological Laboratory of the Free University, Amsterdam, and were kept under standard conditions as described earlier (Dikkeboom *et al.*, 1984). Juvenile (4–5 weeks old) and adult (12–13 weeks old) snails with shell heights of 10 ± 1 mm and 25 ± 1 mm, respectively, were used.

Phagocytic capacity of circulating amoebocytes

Monolayers of circulating amoebocytes were made as described previously (Sminia *et al.*, 1979a) by filling incubation chambers on microscope slides alternately with 10 μl of pooled haemolymph from either 10 juvenile or 10 adult specimens of *L. stagnalis*. After rinsing in snail saline (HEPES buffered, pH 7·8; cf. Sminia *et al.*, 1979a), 200 μl of one of the following suspensions (in snail saline) were added to each chamber: 2% (v/v) formalinized rabbit red blood cells (RRBC) or heat-killed *Staphylococcus saprophyticus* ($\pm 5 \times 10^7$/ml). The slides were incubated horizontally for 15 or 60 min in a moist atmosphere at 20° (bacteria for 60 min only). Then, they were washed carefully in saline and fixed for 5 min by immersion in 100% methanol. The RRBC preparations were stained with haemalum–eosin; the bacterial slides were stained with alcian blue (0·1% in 10% ethanol) and then with 0·1% eosin.

A total of 1000 amoebocytes of each experimental group were studied light-microscopically. In the RRBC slides, the amoebocytes which had phagocytosed 0, 1, 2 or 3-or-more erythrocytes were scored. In the bacterial preparations, only a distinction between phagocytosing and non-phagocytosing cells was made. Moreover, in all preparations, attention was paid to the morphology of the amoebocytes.

Opsonizing activity of snail plasma

Haemolymph from 60 juvenile or adult snails was pooled. In order to prepare plasma, the amoebocytes were removed by centrifugation (250 ***g***, 15 min, 4°). Formalinized RRBC (in snail saline) were pretreated by mixing 200 μl of a 20% erythrocyte suspension with 500 μl plasma of either juvenile or adult snails and 1300 μl snail saline, giving a final RRBC concentration of 2%. These two mixtures and a 2% RRBC suspension in snail saline (control) were incubated for 1 hr at 20° while shaking constantly. The erythrocytes were washed twice and resuspended in 2 ml snail saline.

Meanwhile, monolayers of amoebocytes were prepared as follows: pooled samples were made of haemolymph from 60 juvenile and haemolymph from 60 adult snails; 500 μl of each sample were mixed with 1 ml or 4·5 ml snail saline, respectively, to equalize the cell concentrations (Dikkeboom *et al.*, 1984).

All (8) compartments of 12 Nutacon CL-801 Microprint stock slides were filled with 30 μl amoebocyte suspension, and the slides were left horizontally in a moist atmosphere for 25 min to allow the cells to adhere. The supernatants were poured off, the slides were washed carefully with snail saline, and 30 μl of one of the three RRBC suspensions were added to each compartment. The amoebocytes were left to phagocytose for 15 or 60 min at 20°. The slides were then washed with snail saline, immersed in 100% methanol, stained with haemalum–eosin and mounted in DPX. The percentage of amoebocytes that had phagocytosed 0, 1, 2 and 3-or-more erythrocytes was determined by examining, light-microscopically, 8×100 amoebocytes per slide. Simultaneously, the morphology of the cells was studied.

Agglutinin activity of snail haemolymph

In order to determine the titres of agglutinin in the haemolymph of juvenile and adult snails, the following procedure was used. Of a subpopulation of snails with very reactive agglutinin (type I agglutinin) (Van der Knaap *et al.*, 1982), 26 juvenile snails (10 ± 1 mm) and 26 adult snails (25 ± 1 mm) were selected. A 10 μl sample of extruded haemolymph from each of the 52 snails was mixed with 40 μl of phosphate-buffered saline (PBS, pH 7·8) in the first well of a V-bottomed Greiner microtitre plate, and of these mixtures 25 μl was serially diluted two-fold with 25 μl PBS. Finally, to each well, 25 μl of a suspension of sheep red blood cells (SRBC, 2% in PBS) were added and the titre plates were incubated for 2 hr at 20°.

Each of the 26 juvenile and 26 adult snails was

placed separately in a numbered jar, kept at standard conditions and was fed lettuce *ad libitum*. About 6 weeks later, when the juvenile snails had become adult and had reached a shell height of 25 ± 1 mm, and when the adults had become 30 ± 1 mm, the same procedure to determine the agglutinin titres in the haemolymph was repeated. The titres were expressed as the reciprocals of the highest dilutions at which agglutination occurred.

In vivo *elimination of bacteria*

The method to study the *in vivo* elimination of bacteria by the internal defence system of *L. stagnalis* was a modification of the one used by Van der Knaap *et al.* (1981).

The Gram-positive bacterium *S. saprophyticus* was cultured overnight in nutrient broth. The bacteria were washed and suspended in sterile snail saline. After Bürker-Türk haemocytometer counts, the concentration of the suspension was set at 1×10^8 bacteria per 50 μl. The next day, this suspension was found to contain $6{\cdot}5 \times 10^7$ viable bacteria per 50 μl.

After being anaesthetised in 2% diethylether in conditioned tap water (Meuleman, Huyer & Luub, 1984a), 35 juvenile and 35 adult snails were injected with 5 μl or 50 μl bacterial suspension, respectively. The relation of the injected volumes was based upon the relation of the total body weights of juvenile and adult snails, being approximately 1 to 10. The injections were given in the cephalopedal sinus through glass needles which had an outer diameter of 70–80 μm (cf. Meuleman *et al.*, 1984a). On seven occasions after injection, at $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 4, 8 and 16 hr blood samples were drawn from five snails in each age group (cf. Sminia, 1972). The samples were individually diluted in sterile distilled water, and 50 μl from several dilutions were spread on broth agar plates. The plates were incubated overnight at 37°, and the next day the colonies that had grown were counted. From these counts, the original number of viable bacteria in each blood sample was calculated.

RESULTS

Phagocytic capacity of circulating amoebocytes

After both 15 and 60 min of *in vitro* incubation, the total percentage of amoebocytes of juvenile snails that had phagocytosed RRBC was significantly lower than that of adult snails ($\alpha = 0{\cdot}05$; Fig. 1). For both age groups, the percentage of cells that had phagocytosed

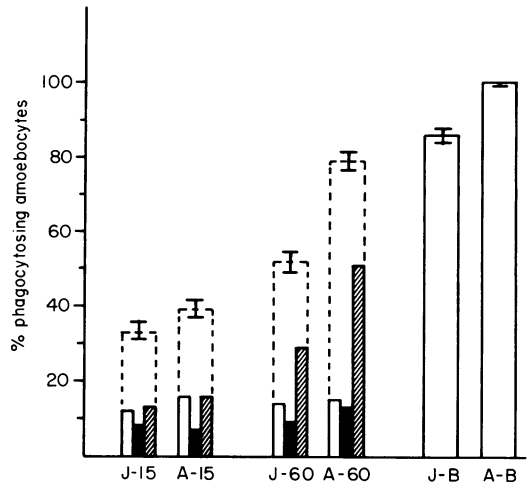

Figure 1. *In vitro* phagocytosis by amoebocytes of juvenile (J) and adult (A) *L. stagnalis*. The first four sets of bars represent the phagocytosis of rabbit red blood cells (RRBC) after 15 or 60 min of incubation. The total percentage (⬚) and the percentages of amoebocytes that have phagocytosed one (□), two (■) or three-or-more (■) RRBC are shown. The last two bars represent the phagocytosis of *Staphylococcus saprophyticus* (B) after 60 min. Means with 95% confidence limits.

was significantly higher after 60 min than after 15 min. This latter difference was mainly caused by an increase in the number of amoebocytes that had taken up 3-or-more RRBC, especially in the adult group.

The percentage of amoebocytes that had phagocytosed bacteria was significantly lower for juvenile snails than it was for adults. For both juveniles and adults, this percentage was significantly higher than the number of cells that had ingested RRBC.

The morphological observations point out that most of the amoebocytes that had not phagocytosed were round (immature) cells (Dikkeboom *et al.*, 1984). On the other hand, all of the amoebocytes that had taken up 3-or-more RRBC were large, spreading (mature) cells.

Opsonizing activity of snail plasma

Pretreatment of RRBC with plasma from juvenile snails barely resulted in an increase in phagocytosis of these particles, as compared to pretreatment with snail saline; pretreatment with plasma from adult snails, however, caused a considerable increase in both the percentage of amoebocytes that phagocytosed and in the number of RRBC that was engulfed per amoebocyte (Fig. 2). The strong opsonic activity of adult

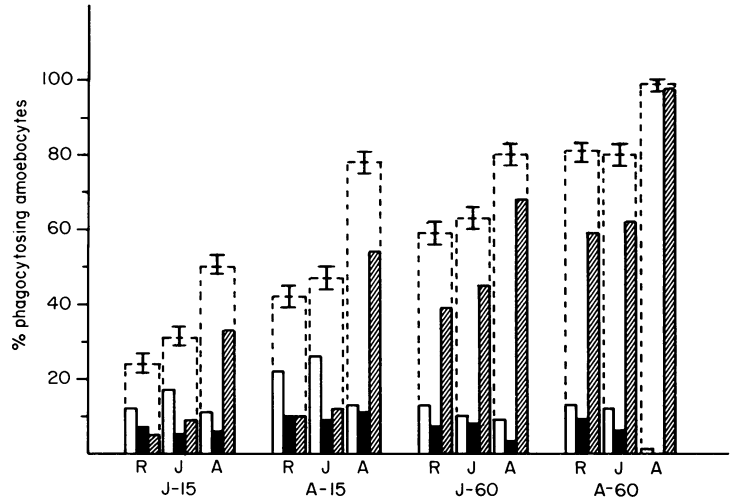

Figure 2. *In vivo* phagocytosis of rabbit red blood cells pretreated with plasma from juvenile (J) or adult (A) *L. stagnalis*, or with snail saline (R) as a control. Amoebocytes of juvenile (J-15, J-60) and adult (A-15, A-60) *L. stagnalis* were incubated for 15 or 60 min. The total percentage of phagocytosing amoebocytes (⬚) and cells with one (□), two (■) or three-or-more (■) RRBC are shown. Means with 95% confidence limits.

plasma was effective towards both juvenile and adult amoebocytes.

Agglutinin activity of snail haemolymph

The agglutinin titres of the haemolymph samples of juvenile snails varied from 40 to 80 when measured against sheep red blood cells (SRBC) *in vitro* (Fig. 3). When these juvenile snails became adult, the haemagglutinin titres increased to a value of about 160, which was the same as the titres found in snails which were adult at the time of the first measurement. The haemolymph of this last group of adult snails showed no increase in haemagglutinin activity as they grew from 25 to 30 mm.

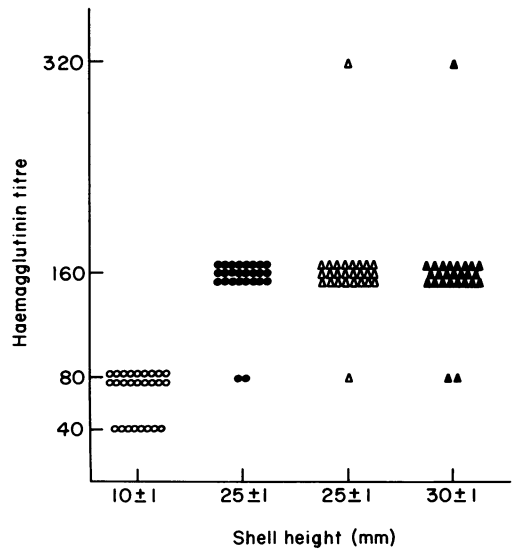

Figure 3. Agglutinin activity of the haemolymph of *L. stagnalis* of different shell heights, measured against sheep red blood cells *in vitro*: (○) juvenile snails (10 mm); (●) same snails, adult (25 mm, 6 weeks later); (△) adult snails (25 mm); (▲) same snails (30 mm, 6 weeks later). Titres are expressed as the reciprocals of the highest dilutions at which agglutination occurred.

In vivo elimination of bacteria

The elimination patterns of *S. saprophyticus* from the circulation of juvenile and adult *L. stagnalis* were more or less identical (Fig. 4). During the first 2 hr after injection, there was a rapid decrease in the number of viable bacteria in the haemolymph. Regression analysis showed that the elimination rate in juvenile snails was slightly ($\alpha = 0{\cdot}1$) slower than in adults only

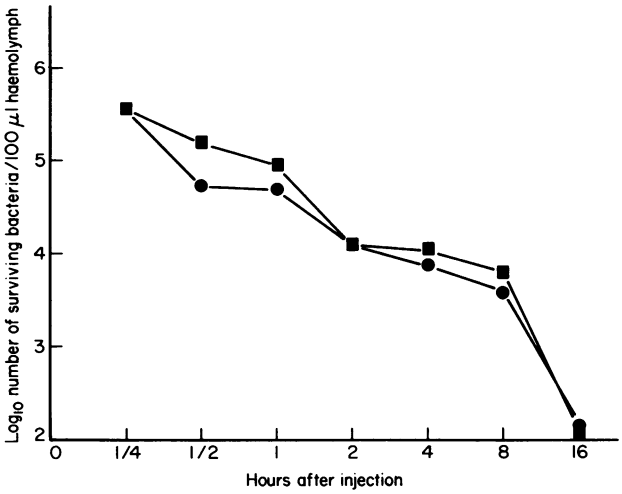

Figure 4. The *in vivo* elimination patterns of *S. saprophyticus* from the haemolymph of (●) juvenile *L. stagnalis* (dose $6{\cdot}5 \times 10^6$ living bacteria) and (■) adult snails (dose $6{\cdot}5 \times 10^7$ living bacteria). Both the number of surviving bacteria and the hours after infection are plotted on logarithmic scales.

between ¼ hr and 2 hr. From 2 to 8 hr, there was no obvious change, whereas from 8 hr on, the number of bacteria decreased again.

DISCUSSION

In a previous paper (Dikkeboom *et al.*, 1984), it was shown that the circulating amoebocytes are both quantitatively and qualitatively less well developed in juvenile *L. stagnalis* than in adult snails. The present study was undertaken to assess whether this lesser development of the amoebocytes, as judged by morphological and enzyme-cytochemical criteria, is also reflected by a functional inferiority of the cells in defence activities. Moreover, it was investigated whether, in addition to cellular defence factors, humoral defence activities of juvenile snails are also less well developed than those of adult specimens.

From the present study, it is obvious that the phagocytic capacity of the circulating amoebocytes of juvenile *L. stagnalis* is lower than that of adult snails. This relative functional incompetence can be correlated to morphological characteristics of the amoebocytes: the non-phagocytosing cells are nearly always the so-called round amoebocytes (Sminia, Van der Knaap & Van Asselt, 1983), and the cells which avidly ingest a larger number of erythrocytes are the spreading cells. This is in agreement with the previous supposition (Dikkeboom *et al.*, 1984) that the round amoebocytes (70% of the circulating amoebocytes in juvenile snails) are less differentiated, immature cells, and that the spreading cells (80% of the adult amoebocytes) are more differentiated and thus more immunocompetent. The fact that about 80% of the juvenile amoebocytes and almost 100% of the adult amoebocytes have phagocytosed bacteria implies that a large number of round amoebocytes displays phagocytic activity. However, the uptake of a foreign particle does not necessarily mean that the phagocytosing cell is able to kill this particle. Thus, it could well be that the round amoebocytes do have the ability to phagocytose bacteria but are not able to kill them intracellularly. Not much is known, however, of the process of intracellular killing by mollusc haemocytes, and this will be a subject of further research. The avidity of amoebocytes of both juvenile and adult snails for bacteria is higher than for erythrocytes. This may be due to the differences in size and physicochemical characteristics of the particles, or both (cf. Sminia *et al.*, 1979a). Still, as was the case with erythrocytes, the percentage of juvenile amoebocytes phagocytosing bacteria was lower than that of adult cells. From these results, it is clear that the phagocytic capacity of a part

of the cellular component of the internal defence system increases in the course of ontogeny, as snails grow from juvenile to adult.

Amoebocytes have been considered to be analogues of mammalian macrophages (Van der Knaap *et al.*, 1983). With respect to the ontogeny of mammalian macrophages, comparable results have been obtained. Vêtvička *et al.* (1982) have shown that a lower percentage of peritoneal macrophages of young mice (10 days) displays phagocytic capacity *in vitro*, and that the number of phagocytosed particles per cell was also lower as compared to adult and aged mice (3–4 and 18 months old, respectively). Blaese & Lawrence (1977) report that the high susceptibility of neonatal rats to an infection with *Listeria monocytogenes* is related to a deficiency in peritoneal macrophage maturity. Injection of macrophages from adult (highly resistant) animals protects the neonatal rat against the infection.

Strong opsonic activity could be detected in the plasma of adult snails, but not in juvenile plasma. However, like adult amoebocytes (Van der Knaap *et al.*, 1983), juvenile cells probably have binding sites for opsonin, as these cells also show a higher phagocytic activity towards adult plasma-treated erythrocytes than towards control RRBC. The fact that opsonic activity could not be detected in juvenile plasma could be due to the four-fold dilution of the plasma used for opsonization. The present observations that even control (i.e. non-opsonized) RRBC are eagerly phagocytosed are in line with a previous study of Sminia *et al.* (1979a) who stated that recognition of foreignness by amoebocytes of adult *L. stagnalis* was facilitated by plasma opsonin, but that it could also take place in the absence of plasma factors.

From the present observations, it is clear that haemolymph from juvenile snails shows a lower agglutinating activity than adult haemolymph. The haemolymph molecules responsible for agglutination have opsonic properties (Renwrantz *et al.*, 1981; Van der Knaap *et al.*, 1982, 1983). It may, therefore, seem surprising that agglutinating activity was found in the haemolymph of juvenile snails, but that this haemolymph seems to lack opsonizing properties. As stated above, (part of) the explanation for this apparent discrepancy may lie in the procedure that was applied. Moreover, it must be borne in mind that, in the test for opsonic activities, the majority of the snails are of subpopulation II, i.e. snails with haemolymph displaying low agglutinating properties (Van der Knaap *et al.*, 1982), whereas in the test for agglutinating activities, all snails were of subpopulation I, with haemolymph displaying very strong agglutinating activities. It has been shown that the opsonic capacity of type II haemolymph is much lower than that of type I haemolymph (Van der Knaap *et al.*, 1983).

The elimination patterns of *S. saprophyticus* in juvenile and adult *L. stagnalis* are more or less identical to the patterns that were found by Van der Knaap *et al.* (1981) for adult snails. There are extremely efficient processes involved in the elimination of these Gram-positive bacteria, as the number of viable *S. saprophyticus* decreases within 2 hr by about 99%. Three of these processes are thought to be agglutination, opsonization and phagocytosis. Juvenile *L. stagnalis* have been shown to be inferior to adults with regard to the agglutinating and opsonizing activity of their haemolymph and the *in vitro* phagocytosis by their amoebocytes. However, their internal defence system is hardly inferior where the elimination of injected bacteria is concerned; regression analysis has shown that the elimination rate is only slightly slower for juvenile snails. A simple explanation for this can be that the dose of injected bacteria was relatively low, and although fewer amoebocytes of juvenile snails are able to phagocytose, and although there is a lesser amount of opsonin/agglutinin present in their haemolymph, this is adequate to eliminate this (small) dose of bacteria quite easily. Other explanations for this apparent contradiction might be that there are processes other than agglutination, opsonization and phagocytosis involved in bacterial elimination. Renwrantz *et al.* (1981) have shown that, in the snail *Helix pomatia*, injected foreign particles are trapped in large numbers in certain blood sinuses before the actual process of phagocytosis occurs. Indeed, this phenomenon also appears to take place in adult *L. stagnalis* (Van der Knaap *et al.*, 1981). Also, cell types other than circulating amoebocytes may play an important role in the elimination of foreign particles. Large numbers of fixed phagocytes (reticulum cells), which are present throughout the connective tissue of adult *L. stagnalis*, are able to ingest bacteria (Sminia *et al.*, 1979b). In addition, a bacteriostatic factor is present in the haemolymph of both juvenile and adult *L. stagnalis* which supresses the viability of *S. saprophyticus* (W. P. W. Van der Knaap, unpublished results).

In conclusion, the present study not only confirms the previous observations that the activities of the cellular component of the internal defence system of juvenile *L. stagnalis* are at a lower level than those of adult snails (Dikkeboom *et al.*, 1984), but also demon-

strates that the activity of humoral defence factors with opsonizing and agglutinating properties is lower. These findings confirm that juvenile *L. stagnalis* have a less well developed, and therefore less competent, immune system than adult snails. This may be an explanation for the fact that juvenile *L. stagnalis* are much more susceptible to infection with the schistosome *Trichobilharzia ocellata* than are adult snails.

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